METHODS AND COMPOSITIONS FOR ASSAYING HOMOCYSTEINE

The present application is a continuation-in-part of U.S. Patent Application Serial No. 09/457,205, filed December 6, 1999, now pending, which is a continuation-in-part of U.S. Patent Application Serial No. 09/347,878, filed July 6, 1999, now allowed. The present application also

5 claims priority benefit of U.S. Provisional Patent Application Serial No. 60/301,895, filed June 29, 2001, now pending. The disclosure of the above-referenced applications is incorporated by reference herein in their entirety.

Technical Field

10 The present invention relates to compositions and methods for assaying homocysteine (Hcy) and its related moieties, e.g., S-adenosylhomocysteine (SAH) or adenosine. More particularly, assay methods that employ, mutant SAH hydrolase having binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, are provided. The modified enzymes and fusion proteins containing the modified enzymes are also provided.

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Background Art

Homocysteine (Hcy) is a thiol-containing amino acid formed from methionine during S-adenosylmethionine-dependent transmethylation reactions. Intracellular Hcy is remethylated to methionine, or is irreversibly catabolized in a series of reactions to form cysteine. Intracellular

20 Hcy is exported into extracellular fluids such as blood and urine, and circulates mostly in oxidized form, and mainly bound to plasma protein (Refsum, et al., Annu. Rev. Medicine, 49:31-62 (1998)). The amount of Hcy in plasma and urine reflects the balance between Hcy production and utilization. This balance may be perturbed by clinical states characterized by genetic disorders of enzymes involved in Hcy transsulfuration and remethylation (e.g., cystathionine ß-synthase and

25 $N^{5,10}$-methylenetetrahydrofolate reductase or dietary deficiency of vitamins (e.g., vitamin B_6, B_{12} and folate) involved in Hcy metabolism (Baual, et al., Cleveland Clinic Journal of Medicine, 64:543-549 (1997)). In addition, plasma Hcy levels may also be perturbed by some medications such as anti-folate drugs (e.g., methotrexate) used for treatments of cancer or arthritis (Foody, et al., Clinician Reviews, 8:203-210 (1998)).

Severe cases of homocysteinemia are caused by homozygous defects in genes encoding for enzymes involved in Hcy metabolisms. In such cases, a defect in an enzyme involved in either Hcy remethylation or transsulfuration leads to as much as 50-fold elevations of Hcy in the blood and urine. The classic form of such a disorder, congenital homocysteinemia (Hcyemia), is caused

5   by homozygous defects in the gene encoding cystathionine ß-synthase (CBS). These individuals suffer from thromboembolic complications at an early age, which result in stroke, myocardial infarction, renovascular hypertension, intermittent claudication, mesenteric ischemic, and pulmonary embolism. Such patients may also exhibit mental retardation and other abnormalities resembling ectopia lentis and skeletal deformities (Perry T., *Homocysteine: Selected aspects in*

10   *Nyham W.L. ed. Heritable disorders of amino acid metabolism.* New York, John Wiley & Sons, pp. 419-451 (1974)). It is also known that elevated Hcy levels in pregnant women is related to birth defects of children with neurotube closures (Scott, *et al., "The etiology of neural tube defects" in Graham, I., Refsum, H., Rosenberg, I.H., and Ureland P.M. ed. "Homocysteine metabolism: from basic science to clinical medicine"* Kluwer Academic Publishers, Boston, pp. 133-136

15   (1995)). Thus, the diagnostic utility of Hcy determinations has been well documented in these clinical conditions.

It has been demonstrated that even mild or moderately elevated levels of Hcy also increase the risk of atherosclerosis of the coronary, cerebral and peripheral arteries and cardiovascular disease (Boushey, *et al., JAMA*, 274:1049-1057 (1995)). The prevalence of Hcyemia was shown

20   to be 42%, 28%, and 30% among patients with cerebral vascular disease, peripheral vascular disease and cardiovascular disease, respectively (Moghadasian, *et al., Arch. Intern. Med.*, 157:2299-2307 (1997)). A meta-analysis of 27 clinical studies calculated that each increase of 5 µM in Hcy level increases the risk for coronary artery disease by 60% in men and by 80% in women, which is equivalent to an increase of 20 mg/dl^{-1} (0.5 mmol/dl^{-1}) in plasma cholesterol,

25   suggesting that Hcy, as a risk factor, is as strong as cholesterol in the general population. Results from these clinical studies concluded that hyperhomocysteinemia is an emerging new independent risk factor for cardiovascular disease, and may be accountable for half of all cardiovascular patients who do not have any of the established cardiovascular risk factors (*e.g.*, hypertension, hypercholesterolemia, cigarette smoking, diabetes mellitus, marked obesity and physical

30   inactivity).

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Mild homocysteinemia is mainly caused by heterozygosity of enzyme defects. A common polymorphism in the gene for methylenetetrahydrofolate reductase appears to influence the sensitivity of homocysteine levels to folic acid deficiency (Boers, *et al., J. Inher. Metab. Dis.*, 20:301-306 (1997)). Moreover, plasma homocysteine levels are also significantly increased in

5 heart and renal transplant patients (Ueland, *et al., J. Lab. Clin. Med.*, 114:473-501 (1989)), Alzheimer patients(Jacobsen, *et al., Clin. Chem.*, 44:2238-2239 (1998)), as well as in patients of non-insulin-dependent diabetes mellitus (Ducloux, *et al., Nephrol. Dial. Transplantl*, 13:2890-2893 (1998)). The accumulating evidence linking elevated homocysteine with cardiovascular disease has prompted the initiation of double-blind, randomized and placebo controlled multicenter

10 clinical trials to demonstrate the efficacy of lowering plasma Hcy in preventing or halting the progress of vascular disease (Diaz-Arrastia, *et al., Arch. Neurol.*, 55:1407-1408 (1998)). Determination of plasma homocysteine levels should be a common clinical practice.

As a risk factor for cardiovascular disease, the determination of total plasma Hcy levels (reduced, oxidized and protein-bound) has been recommended in clinical setting (Hornberger, *et*

15 *al., American J. of Public Health*, 88:61-67 (1998)). Since 1982, several methods for determining total plasma Hcy have been described (Mansoor, *et al., Anal. BioChem.*, 200:218-229 (1992); Steir, *et al., Arch. Intern. Med.*, 158:1301-1306 (1998); Ueland, *et al., Clin. Chem.*, 39:1764-1779 ()1993); and Ueland, *et al., "Plasma homocysteine and cardiovascular disease" in Francis, R.B.Jr.eds. Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function.* New

20 York, Marcel Dokker, pp. 183-236 (1992); see, also, Ueland, *et al., "Plasma homocysteine and cardiovascular disease" in Francis, R.B.Jr.eds. Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function.* New York, Marcel Dokker, pp. 183-236 (1992)). The assay of total Hcy in plasma or serum is complicated by the fact that 70% of plasma Hcy is protein-bound and 20-30% exists as free symmetric or mostly asymmetric mixed disulfides. Free

25 reduced Hcy exists in only trace amounts (Stehouwer, *et al., Kidney International*, 55308-314 (1999)).

Most of the methods require sophisticated chromatographic techniques such as HPLC, capillary gas chromatography, or mass spectrometry (GC/MS) to directly or indirectly (*e.g.*, enzymatic conversion of Hcy to SAH (S-adenosylhomocysteine) by SAH hydrolase followed by

30 HPLC or TLC separation) measure Hcy. Radioenzymatic conversion of Hcy to radiolabeled SAH

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by SAH hydrolase prior to TLC separation has also been used. In these assays, chromatographic separation, which is often time-consuming and cumbersome to perform, is a common key step of these methods. More particularly, these methods require highly specialized and sophisticated equipment and well-trained analytic specialists. The use of such equipment is generally not well-

5   accepted in routine clinical laboratory practice.

Immunoassays for Hcy that use a monoclonal antibody against SAH (Araki, *et al.*, *J. Chromatog.*, <u>422</u>:43-52 (1987)) are also known. These assays are based upon conversion of Hcy to SAH, which is then detected by a monoclonal antibody. Monoclonal antibody against albumin-bound Hcy has been developed for determination of albumin-bound Hcy (Stabler, *et al.*, *J. Clin.*

10   *Invest.*, <u>81</u>:466-474 (1988)), which is the major fraction of total plasma Hcy. Other immunological protocols are also available (see, *e.g.*, U.S. Patent Nos. 5,631,127, 5,827,645, 5,958,717, 6,063,581 and 5,885,767). Though immunoassays avoid a time-consuming chromatographic separation step and are amenable to automation, production of monoclonal antibody is expensive, somewhat unpredictable, and often requires secondary or even tertiary antibodies for detection.

15   It is an object herein to provide assays for quantifying and/or detecting homocysteine in body fluids and body tissues.

Disclosure of the Invention

Assays that employ mutant SAH hydrolase having binding affinity for Hcy, SAH or

20   adenosine but has attenuated catalytic activity are provided. These methods are designated substrate trapping methods; and the modified SAH hydrolases, are designated as "substrate trapping SAH hydrolases." The substrate trapping SAH hydrolases and methods for preparing them are also provided. The assays can be used for prognostic, diagnostic, drug screening or treatment monitoring purposes.

25   The assays readily can be automated. In addition, the assays can be adapted for use in point of care systems and in home test kits. For example, blood test point of care systems can be adapted for measuring homocysteine levels using the mutant SAH hydrolases provided herein. Home test kits may also be adapted for use with the methods and mutant SAH hydrolases provided herein.

In one aspect, the present invention is directed to a method for assaying homocysteine (Hcy), S-adenosylhomocysteine (SAH) or adenosine in a sample, which method comprises: a) contacting a sample containing or suspected of containing Hcy, SAH or adenosine with a mutant SAH hydrolase, wherein said mutant SAH hydrolase has binding affinity for Hcy, SAH or

5   adenosine but has attenuated catalytic activity, and said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) detecting binding between Hcy, SAH or adenosine with said mutant SAH hydrolase, whereby the presence or amount of Hcy, SAH or adenosine in said sample is assessed.

10   Any suitable mutant SAH hydrolases can be used in the present methods. In one example, the mutant SAH hydrolase used in the method has a mutation in an amino acid residue that is directly involved in the SAH hydrolase's catalytic activity, its binding with NAD^+, NADH, Hcy, SAH or adenosine. In another example, the mutant SAH hydrolase used in the method has a mutation in an amino acid residue that is adjacent to an amino acid residue that is directly involved

15   in the SAH hydrolase's catalytic activity, its binding with NAD^+, NADH, Hcy, SAH or adenosine.

The mutant SAH hydrolase can have enhanced binding affinity for Hcy, SAH or adenosine than a wild type SAH hydrolase from which said mutant SAH hydrolase is derived. Preferably, the mutant SAH hydrolase has at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 fold higher binding affinity for Hcy, SAH or adenosine than a wild type SAH hydrolase from which said mutant SAH

20   hydrolase is derived.

The mutant SAH hydrolase can be derived from any suitable sources. For example, the mutant SAH hydrolase can be derived from a mammalian SAH hydrolase, *e.g.*, derived from a human SAH hydrolase.

In a specific embodiment, the mutant SAH hydrolase used in the method comprises the

25   amino acid sequence set forth in SEQ ID NO:1 and comprises a mutation such as R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S,

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L409G, S420T, P424A, F425S, P427A, D428G, H429A, Y430T, R431K, R431G, Y432S, Y432A or Y432F, or a combination thereof.

Prior to the contact between the sample and the mutant SAH hydrolase, oxidized or conjugated Hcy in the sample can be converted into reduced Hcy. Similarly, prior to the contact

5   between the sample and the mutant SAH hydrolase, the Hcy in the sample can be converted into SAH. Preferably, oxidized or conjugated Hcy in the sample is converted into reduced Hcy and then the reduced Hcy is converted into SAH.

Hcy in the sample can be converted into SAH by any suitable method, e.g., by a wild-type SAH hydrolase and access adenosine. Preferably, the access adenosine in the sample is removed

10   by adenosine deaminase while the wild-type SAH hydrolase is inhibited. Any suitable SAH hydrolase inhibitors can be used, e.g., neplanocin A or aristeromycin.

The present method can further comprise a step of removing the reducing agent used to convert oxidized or conjugated Hcy into reduced Hcy prior to or concurrently with contacting the sample with the mutant SAH hydrolase. The reducing agent can be removed by any suitable

15   methods such as chromatography. Exemplary chromatography includes column, paper, thin layer and batch chromatography. Preferably, the reducing agent is removed by batch chromatography. The removal of the reducing agent can have certain benefits such as increasing assay sensitivity. The degree of the removal is affected by a number of factors including the reducing agent used, the removal methods used and the objective of the removal. Although the reducing agent may be

20   substantially removed, it is often not necessary to do so; for a 10-50 percent reduction of the reducing agent can bring significant benefits to the assay.

An indicator dye can be used in the present method for various reasons, e.g., for the ease of monitoring sample and reagent addition and transfer. The present method can further comprise a step of removing the indicator dye prior to or concurrently with contacting the sample with the

25   mutant SAH hydrolase. The indicator dye can be removed by any suitable methods such as chromatography. Exemplary chromatography includes column, paper, thin layer and batch chromatography. Preferably, the indicator dye is removed by batch chromatography. The degree of the removal is affected by a number of factors including the indicator dye used, the removal methods used and the objective of the removal. Although the reducing agent may be substantially

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removed, it is often not necessary to do so; for a 10-70 percent reduction of the indicator dye can bring significant benefits to the assay.

In a specific embodiment, the reducing agent and the indicator dye are removed by the same method, *e.g.*, chromatography. Preferably, the reducing agent and the indicator dye are

5   removed by a batch chromatography.

The present method can be conducted in any suitable format, *e.g.*, in a competitive or sandwich format, in solution or on a solid support. In a specific embodiment, the SAH is contacted with the mutant SAH hydrolase in the presence of a labeled SAH or a derivative or an analogue thereof, thereby the amount of the mutant SAH hydrolase bound to the labeled SAH inversely

10   relates to the amount of SAH in the sample. Any suitable labels can be used such as chemical, radioactive, enzymatic, fluorescent or luminescent label. Preferably, the labeled SAH or a derivative or an analogue thereof is fluorescently, enzymatically or proteinaceously labeled. For example, the fluorescently labeled SAH can be fluorecin-SAH conjugate or Rocamin-SAH conjugate, said fluorecin or Rocamin being linked to said SAH or a derivative or an analogue

15   thereof by a linker of 1-15 carbon atom length; the enzymatically labeled SAH derivative can be Glucose-6-phosphate dehydrogenase (G-6-PDH-SAH) conjugate, alkaline phosphatase-SAH conjugate, or malate dehydrolase-SAH conjugate, said G-6-PDH, alkaline phosphatase or malate dehydrolase being linked to said SAH or a derivative or an analogue thereof by a linker of 1-15 carbon atom length; and the proteinaceously labeled SAH derivative can be bovine albumin-SAH

20   conjugate, said bovine albumin being linked to said SAH or a derivative or an analogue thereof by a linker of 1-15 carbon atom length.

In another specific embodiment, the mutant SAH hydrolase is a labeled mutant SAH hydrolase. Any suitable labels can be used such as chemical, radioactive, enzymatic, fluorescent or luminescent label. Preferably, the labeled mutant SAH hydrolase is a fluorescently,

25   enzymatically, biotin or streptavidin labeled mutant SAH hydrolase. For example, the biotin labeled mutant SAH hydrolase is detected by a streptavidin labeled enzyme; the streptavidin labeled enzyme is a streptavidin labeled horse radish phosphatase (HRP).

The labeled SAH or a derivative or an analogue thereof, or the labeled mutant SAH hydrolase can be immobilized on a surface suitable for conducting an assay for Hcy or its related

30   moieties. For example, the bovine albumin-SAH conjugate can be immobilized.

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In a specific assay format, the fluorescently labeled SAH or a derivative or an analogue thereof is directly contacted by the mutant SAH hydrolase, and the resulting change of fluorescent polarization is measured for assessing Hcy, SAH or adenosine. In another specific assay format, the enzymatically labeled SAH or a derivative or an analogue thereof is directly contacted by the

5 mutant SAH hydrolase, and the resulting change of enzyme activity is measured for assessing Hcy, SAH or adenosine.

The present method can be used to assay any sample, *e.g.*, a body fluid or a biological tissue. Exemplary body fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus and amniotic fluid. Preferably, the body fluid to be assayed is

10 blood. The blood sample can be assayed directly or be treated before assaying. For example, the blood sample can be further separated into a plasma or serum fraction.

The present method can be used alone, or can be used in combination with other related assays. For example, the present method can further comprise a step of detecting cholesterol and/or folic acid in the sample, whether sequentially or simultaneously.

15 In another aspect, the present invention is directed to a combination, which combination comprises: a) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) reagents

20 for detecting binding between Hcy, SAH or adenosine and said SAH hydrolase. The combination can further comprise a reagent for detecting cholesterol and/or folic acid.

A kit comprising the above combination is also provided. The kit can further comprise instructions for assaying Hcy, SAH or adenosine in a sample.

In still another aspect, the present invention is directed to an article of manufacture, which

25 article of manufacture comprises: a) packaging material; b) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and c) a label indicating that the mutant SAH

30 hydrolase and the means for use in assaying Hcy, SAH or adenosine in a sample.

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In yet another aspect, the present invention is directed to an isolated nucleic acid fragment, which isolated nucleic acid fragment comprises a sequence of nucleotides encoding a mutant SAH hydrolase, wherein said mutant SAH hydrolase comprises the amino acid sequence set forth in SEQ ID NO:1 or a nucleotide sequence set forth in SEQ ID NO:2 and comprises one or more of

5   the following mutations: R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S, L409G, S420T, P424A, F425S, P427A, D428G, H429A, Y430T,

10   R431K, R431G, Y432S, Y432A, Y432F.

The isolated nucleic acid fragment can be in any suitable forms, *e.g.*, DNA, RNA, PNA, etc., or a combination thereof. A plasmid comprising the above isolated nucleic acid fragment is also provided. A cell comprising the above plasmid is also provided. Any suitable cells can be used. For example, the cell can be a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect

15   cell or an animal cell. A method for producing a mutant SAH hydrolase is also provided, which method comprises growing the above cell under conditions whereby the mutant SAH hydrolase is expressed by the cell, and recovering the expressed mutant SAH hydrolase.

In yet another aspect, the present invention is directed to a substantially purified mutant SAH hydrolase, wherein said mutant SAH hydrolase comprises the amino acid sequence set forth

20   in SEQ ID NO:1 and comprises one or more of the following mutations: R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S,

25   L409G, S420T, P424A, F425S, P427A, D428G, H429A, Y430T, R431K, R431G, Y432S, Y432A, Y432F.

In yet another aspect, the present invention is directed to a conjugate, which conjugate comprises: a) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity

30   of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its

binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) a facilitating agent linked to the mutant SAH hydrolase directly or via a linker, wherein the agent facilitates: i) affinity isolation or purification of a conjugate; ii) attachment of a conjugate to a surface; or iii) detection of a conjugate. The conjugate can be a fusion protein. Alternatively, the

5   mutant SAH hydrolase and the facilitating agent can be linked by other means.

Brief Description of the Drawings

Fig. 1 depicts Hcy assay using wild type and mutant SAH hydrolase.

Fig. 2 depicts total plasma Hcy assay procedure with wild type and mutant SAH hydrolase.

10   Fig. 3 depicts design and synthesis of fluorescence labeled tracer.

Fig. 4 depicts selection of mutant SAH hydrolase that lacks catalytic activity but retains substrate binding affinity.

Fig. 5 shows Kd values for various SAH hydrolase mutants.

15 Modes of Carrying Out the Invention

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

A.   Definitions

20   Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published

25 applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

As used herein, "a" or "an" means "at least one" or "one or more."

As used herein, "homocysteine (Hcy)" refers to a compound with the following molecular formula: $HSCH_2CH_2CH(NH_2)COOH$. Biologically, Hcy is produced by demethylation of

30 methionine and is an intermediate in the biosynthesis of cysteine from methionine. The term

"Hcy" encompasses free Hcy (in the reduced form) and conjugated Hcy (in the oxidized form). Hcy can conjugate with proteins, peptides, itself or other thiols through disulfide bond.

As used herein, "SAH hydrolase" refers to an ubiquitous eukaryotic enzyme, which is also found in some prokaryotes, which catalyzes hydrolysis of SAH to adenosine (Ado) and Hcy. SAH hydrolase also catalyzes the formation of SAH from Ado and Hcy. The co-enzyme of SAH hydrolase is $NAD^+/NADH$. SAH hydrolase may have several catalytic activities. In the hydrolytic direction, the first step involves oxidation of the 3'-hydroxyl group of SAH (3'-oxidative activity) by enzyme-bound NAD^+ ($E-NAD^+$), followed by ß-elimination of L-Hcy to give 3'-keto-4',5'-didehydro-5'-deoxy-Ado. Michael addition of water to the 5'-position to this tightly bound intermediate (5'-hydrolytic activity) affords 3'-keto-Ado, which is then reduced by enzyme-bound NADH (E-NADH) to Ado (3'-reduction activity). It is intended to encompass SAH hydrolase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "mutant SAH hydrolase, wherein said mutant SAH hydrolase has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity" refers to a mutant form of SAH hydrolase that retains sufficient binding affinity for Hcy, SAH or adenosine to be detected in the process or method, particularly assay, of interest. Typically this is at least about 10%, preferably at least about 50% binding affinity for Hcy, SAH or adenosine, compared to its wildtype counterpart SAH hydrolase. Preferably, such mutant SAH hydrolase retains 60%, 70%, 80%, 90%, 100% binding affinity for Hcy, SAH or adenosine compared to its wildtype counterpart Hcy, SAH or adenosine, or has a higher binding affinity than its wildtype counterpart Hcy, SAH or adenosine. Such mutant Hcy, SAH or adenosine can be herein referred to as a "substrate trapping Hcy, SAH or adenosine," *i.e.*, a molecule that specifically binds to Hcy, SAH or adenosine, but does not catalyze conversion therebetween.

As used herein, a conjugate refers to the compounds provided herein that include one or more mutant analyte-binding enzymes, *e.g.*, mutant SAH hydrolase, and one or more facilitating agents. These conjugates include those produced by recombinant means as fusion proteins, those produced by chemical means, such as by chemical coupling, through, for example, coupling to sulfhydryl groups, and those produced by any other method whereby at least one mutant analyte-binding enzyme is linked, directly or indirectly via linker(s) to a facilitating agent.

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As used herein, a facilitating agent is any moiety, such as a protein or effective portion thereof, that promotes or facilitates, for example, preferably:

i)   affinity isolation or purification of the conjugate;

ii)   attachment of the conjugate to a surface; or

5   iii)   detection of the conjugate or complexes containing the conjugate.

As used herein the term "assessing" is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the amount or concentration of the analyte, *e.g.*, a homocysteine co-substrate, present in the sample, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of analyte in the sample. Assessment 10 may be direct or indirect and the chemical species actually detected need not of course be the analyte itself but may for example be a derivative thereof or some further substance.

As used herein, "attenuated catalytic activity" refers to a mutant SAH hydrolase that retains sufficiently reduced catalytic activity to be useful in the present method. The precise reduction in catalytic activity for use in the assays can be empirically determined for each assay. Typically, the 15 enzyme will retain less than about 50% of one of its catalytic activities or less than 50% of its overall catalytic activities compared to its wildtype counterpart. Preferably, a mutant SAH hydrolase retains less than 40%, 30%, 20%, 10%, 1%, 0.1%, or 0.01% of one of its catalytic activities or its overall catalytic activities compared to its wildtype counterpart. More preferably, a mutant SAH hydrolase lacks detectable level of one of its catalytic activities or its overall catalytic 20 activities compared to its wildtype counterpart. In instances in which catalytic activity is retained and/or a further reduction thereof is desired, the contacting step can be effected in the presence of a catalysis inhibitor. Such inhibitors, include, but are not limited to, heavy metals, chelators or other agents that bind to a co-factor required for catalysis, but not for binding, and other such agents.

25   As used herein, "SAH hydrolase catalysis inhibitor" refers to an agent that inhibits one or all of SAH hydrolase catalytic activities, *e.g.*, 3'-oxidative activity, 5'-hydrolytic activity, or 3'-reduction activity, while not affecting SAH hydrolase's binding affinity for Hcy and/or SAH.

As used herein, adenosine deaminase" refers to an enzyme that catalyzes the deamination of adenosine to form inosine. It is intended to encompass adenosine deaminase with conservative 30 amino acid substitutions that do not substantially alter its activity.

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As used herein, "folate species" refers to folate or folic acid, which is chemically N-[4-[[2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzxoyl]-L-glutamic acid, or a derivative thereof. Examples of folate derivatives include, but are not limited to, dihydrofolate, tetrahydrofolate, 5,-methyl-tetrahydrofolate and 5,10-methylene tetrahydrofolate.

5   As used herein, "serum" refers to the fluid portion of the blood obtained after removal of the fibrin clot and blood cells, distinguished from the plasma in circulating blood.

As used herein, "plasma" refers to the fluid, noncellular portion of the blood, distinguished from the serum obtained after coagulation.

As used herein, "substantially pure" means sufficiently homogeneous to appear free of
10   readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce
15   substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, "antibody" includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.
20   As used herein, "production by recombinant means" refers to production methods that use recombinant nucleic acid methods that rely on well known methods of molecular biology for expressing proteins encoded by cloned nucleic acids.

As used herein, "complementary" when referring to two nucleic acid molecules, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more
25   preferably with less than 15%, even more preferably with less than 5%, most preferably with no mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

As used herein: "stringency of hybridization" in determining percentage mismatch is as follows:
30   1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C;

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2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C (also referred to as moderate stringency); and

3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term "substantially" identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, a "composition" refers to any mixture of two or more products or compounds. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a "combination" refers to any association between two or among more items.

As used herein, "fluid" refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, "vector (or plasmid)" refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well known within the skill of the artisan. An expression vector includes vectors capable of expressing DNA's that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, "a promoter region or promoter element" refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the

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promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in

5   prokaryotes include the bacteriophage T7 and T3 promoters, and the like.

As used herein, "operatively linked or operationally associated" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between

10   the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either

15   at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak, *J. Biol. Chem.*, 266:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, "sample" refers to anything which may contain an analyte for which an

20   analyte assay is desired. The sample may be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure,

25   including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s).

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972)

30   *Biochem.* 11:1726).

As used herein, "protein binding sequence" refers to a protein or peptide sequence that is capable of specific binding to other protein or peptide sequences generally, to a set of protein or peptide sequences or to a particular protein or peptide sequence.

As used herein, "epitope tag" refers to a short stretch of amino acid residues corresponding to an epitope to facilitate subsequent biochemical and immunological analysis of the "epitope tagged" protein or peptide. "Epitope tagging" is achieved by appending the sequence of the "epitope tag" to the protein-encoding sequence in an appropriate expression vector. "Epitope tagged" proteins can be affinity purified using highly specific antibodies raised against the tags.

As used herein, "Protein A or Protein G" refers to proteins that can bind to Fc region of most IgG isotypes. Protein A or Protein G are typically found in the cell wall of some strains of *staphylococci*. It is intended to encompass Protein A or Protein G with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "nucleotide binding sequence" refers to a protein or peptide sequence that is capable of specific binding to nucleotide sequences generally, to a set of nucleotide sequences or to a particular nucleotide sequence.

As used herein, "lipid binding sequence" refers to a protein or peptide sequence that is capable of specific binding to lipids generally, to a set of lipids or to a particular lipid.

As used herein, "polysaccharide binding sequence" refers to a protein or peptide sequence that is capable of specific binding to polysaccharides generally, to a set of polysaccharides or to a particular polysaccharide.

As used herein, "metal binding sequence" refers to a protein or peptide sequence that is capable of specific binding to metal ions generally, to a set of metal ions or to a particular metal ion.

As used herein, "alkaline phosphatases" refers to a family of functionally related enzymes named after the tissues in which they predominately appear. Alkaline phosphatases carry out hydrolase/transferase reactions on phosphate-containing substrates at a high pH optimum. It is intended to encompass alkaline phosphatases with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "glutathione S-transferase" refers to a ubiquitous family of enzymes with dual substrate specificities that perform important biochemical functions of xenobiotic

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biotransformation and detoxification, drug metabolism, and protection of tissues against peroxidative damage. The basic reaction catalyzed by glutathione S-transferase is the conjugation of an electrophile with reduced glutathione (GSH) and results in either activation or deactivation/detoxification of the chemical. It is intended to encompass a glutathione S-transferase

5   with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, high-throughput screening (HTS) refers to processes that test a large number of samples, such as samples of diverse chemical structures against disease targets to identify "hits" (see, *e.g.*, Broach, *et al.*, High throughput screening for drug discovery, *Nature, 384*:14-16 (1996); Janzen, *et al.*, High throughput screening as a discovery tool in the

10   pharmaceutical industry, *Lab Robotics Automation: 8261-265* (1996); Fernandes, P.B., Letter from the society president, *J. Biomol. Screening, 2*:1 (1997); Burbaum, *et al.*, New technologies for high-throughput screening, *Curr. Opin. Chem. Biol., 1*:72-78 (1997)]. HTS operations are highly automated and computerized to handle sample preparation, assay procedures and the subsequent processing of large volumes of data.

15   As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from, *e.g.*, infection or genetic defect, and characterized by identifiable symptoms.

B.   Methods for assaying Hcy, SAH or adenosine

In one aspect, the present invention is directed to a method for assaying homocysteine

20   (Hcy), S-adenosylhomocysteine (SAH) or adenosine in a sample, which method comprises: a) contacting a sample containing or suspected of containing Hcy, SAH or adenosine with a mutant SAH hydrolase, wherein said mutant SAH hydrolase has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, and said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's

25   catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) detecting binding between Hcy, SAH or adenosine with said mutant SAH hydrolase, whereby the presence or amount of Hcy, SAH or adenosine in said sample is assessed.

Nucleic acids encoding SAH hydrolase

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Nucleic acids encoding SAH hydrolase can be obtained by methods known in the art. Additional nucleic acid molecules encoding such enzymes are known and the molecules or sequences thereof are publicly available. If the molecules are available they can be used; alternatively the known sequences can be used to obtain clones from selected or desired sources.

5 For example, the nucleic acid sequences of SAH hydrolases can be used in isolating nucleic acids encoding SAH hydrolases from natural sources. Alternatively, nucleic acids encoding SAH hydrolases can be obtained by chemical synthesis according to the known sequences.

In one embodiment, the nucleic acid molecules containing sequences of nucleotides with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding SAH

10 hydrolase: AF129871 (Gossypium hirsutum); AQ003753 (Cryptosporidium parvum); AF105295 (Alexandrium fundyense); AA955402 (Rattus norvegicus); AA900229 (Rattus norvegicus); AA874914 (Rattus norvegicus); AA695679 (Drosophila melanogaster ovary); AA803942 (Drosophila melanogaster ovary; AI187655 (Manduca sexta male antennae); U40872 (Trichomonas vaginalis); AJ007835 (Xenopus Laevis); AF080546 (Anopheles gambiae);

15 AI069796 (T. cruzi epimastigote); Z97059 (Arabidopsis thaliana); AF059581 (Arabidopsis thaliana); U82761 (Homo sapiens); AA754430 (Oryza sativa); D49804 (Nicotiana tabacum); D45204 (Nicotiana tabacum); X95636 (D. melanogaster); T18277 (endosperm Zea mays); R75259 (Mouse brain); Z26881 (C. roseus); X12523 (D. discoideum); X64391 (Streptomyces fradiae); W21772 (Maize Leaf); AH003443 (Rattus norvegicus); U14963 (Rattus norvegicus); U14962

20 (Rattus norvegicus); U14961 (Rattus norvegicus); U14960 (Rattus norvegicus); U14959 (Rattus norvegicus); U14937 (Rattus norvegicus); U14988 (Rattus norvegicus); U14987 (Rattus norvegicus); U14986 (Rattus norvegicus); U14985 (Rattus norvegicus); U14984 (Rattus norvegicus); U14983 (Rattus norvegicus); U14982 (Rattus norvegicus); U14981 (Rattus norvegicus); U14980 (Rattus norvegicus); U14979 (Rattus norvegicus); U14978 (Rattus

25 norvegicus); U14977 (Rattus norvegicus); U14976 (Rattus norvegicus); U14975 (Rattus norvegicus); L32836 (Mus musculus); L35559 (Xenopus laevis); Z19779 (Human foetal Adrenals tissue); L23836 (Rhodobacter capsulatus); M15185 (Rat); L11872 (Triticum aestivum); M19937 (Slime mold (D. discoideum); M80630 (Rhodobacter capsulatus). Preferably, the nucleic acid molecules containing nucleotide sequences with the GenBank accession Nos. M61831-61832 can

30 be used in obtaining nucleic acid encoding SAH hydrolase (SEQ ID No. 1; see also Coulter-Karis

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and Hershfield, *Ann. Hum. Genet.*, 53(2):169-175 (1989)). Also preferably, the nucleic acid molecule containing the sequence of nucleotides or encoding the amino acids set forth in SEQ ID No. 3 can be used (see also U.S. Patent No. 5,854,023).

5   Selecting and producing Hcy-binding enzymes

Once nucleic acids encoding SAH hydrolases are obtained, these nucleic acids can be mutagenized and screened and/or selected for mutant SAH hydrolase having binding affinity for Hcy, SAH or adenosine but having attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be introduced into nucleic acids encoding SAH hydrolases according to methods

10   known to those of skill in the art. Information regarding the structural-functional relationship of the SAH hydrolases can be used in the mutagenesis and selection of mutant SAH hydrolase having binding affinity for Hcy, SAH or adenosine but having attenuated catalytic activity.

In one example, the mutant SAH hydrolase used in the method has a mutation in an amino acid residue that is directly involved in the SAH hydrolase's catalytic activity, its binding with

15   NAD^+, NADH, Hcy, SAH or adenosine. In another example, the mutant SAH hydrolase used in the method has a mutation in an amino acid residue that is adjacent to an amino acid residue that is directly involved in the SAH hydrolase's catalytic activity, its binding with NAD^+, NADH, Hcy, SAH or adenosine. Information on the SAH hydrolase's catalytic domain, various binding domains including the NAD binding domain and conserved amino acid residues are generally

20   known and can be used in the designing of a suitable mutant SAH hydrolase (*See e.g.*, Turner et al., *Nat. Struct. Biol.*, 5(5):369-76 (1998) entitled "Structure determination of selenomethionyl S-adenosylhomocysteine hydrolase using data at a single wavelength;" Yin et al., *Biomedical Chemistry: Applying Chemical Principles to the Understading and Treatetment of Diesease (Ed. Torrence)*, Chapter 2, Mechanism-based S-adenosylhomocysteine hydrolase inhibitors in the

25   saerch for broad-spectrum antiviral agents), John Wiley & Sons, Inc. (2000); Hu et al., *Biochemistry*, 38(26):8323-33 (1999) entitled "Crystal structure of S-adenosylhomocysteine hydrolase from rat liver;" Creedon et al., *J. Biol. Chem.*, 269(23):16364-70 (1994) entitled "Plasmodium falciparum S-adenosylhomocysteine hydrolase. cDNA identification, predicted protein sequence, and expression in Escherichia coli.;" and Henderson et al., *Mol. Biochem.*

Parasitol., 53(1-2):169-83 (1992) entitled "Cloning of the gene encoding Leishmania donovani S-adenosylhomocysteine hydrolase, a potential target for antiparasitic chemotherapy."

Once a mutant SAH hydrolase with desired properties, *i.e.*, substantially retaining binding affinity for Hcy, SAH or adenosine but having attenuated catalytic activity, is identified, such mutant SAH hydrolase can be produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof. Preferably, the mutant SAH hydrolase is obtained by recombinant expression.

Mutant SAH hydrolase and nucleic acids encoding the mutant SAH hydrolase

SAH hydrolase from mammalian sources are homotetramer of approximate molecular weight of 180-190 KD. The enzyme contains 4 molecules of tightly-bound NAD^+ as a co-enzyme. The catalytic mechanism of the enzyme in the hydrolytic direction includes two consecutive reactions, *i.e.*, the 3'-oxidation of the substrate to 3'-keto in concomitant with the reduction of the enzyme-bound NAD^+ to NADH, and followed by the 5'-hydrolysis to release the reaction products Hcy and Ado (Refsum, *et al.*, *Clin. Chem.*, 31:624-628 (1985)). The C-terminal regions of all known SAH hydrolase are extremely conserved and contain essential amino acid residues to the enzyme catalysis. The crystal structure of human SAH hydrolase in complex with a substrate analog inhibitor was recently determined. This x-ray structure of SAH hydrolase indicates that at least twenty amino acid residues are directly or indirectly interacting with the substrate analog inhibitor and co-enzyme NAD^+. Mutations of those amino acid residues that are involved directly or indirectly in the substrate binding and catalysis can readily be made by site-directed mutagenesis, and the sequence of the resulting mutant enzyme can be confirmed by comparing the mutant SAH hydrolase DNA sequence with the sequence of the wild type enzyme to ensure no other mutations are introduced to the specific mutant enzyme.

Provided herein is a substantially purified mutant SAH hydrolase that substantially retains its binding affinity or has enhanced binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity.

In one specific embodiment, the attenuated catalytic activity of the mutant SAH hydrolase is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof.

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In another specific embodiment, the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

In still another specific embodiment, the mutant SAH hydrolase irreversibly binds SAH.

In yet another specific embodiment, the mutant SAH hydrolase has a Km for SAH that is about or less than 10.0 μM. Preferably, the mutant SAH hydrolase has a Km for SAH that is about 1.0 μM or less than 1.0 μM.

In yet another specific embodiment, the mutant SAH hydrolase has a Kcat for SAH that is about or less than 0.1 S^{-1}.

In yet another specific embodiment, the mutant SAH hydrolase has one or more insertion, deletion, or point mutation(s). Preferably, the mutant SAH hydrolase is derived from the sequence of amino acids set forth in SEQ ID NO:1 or encoded by the sequence of nucleotides set forth in SEQ ID NO:2 but has one or more of the following mutations: R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S, L409G, S420T, P424A, F425S, P427A, D428G, H429A, Y430T, R431K, R431G, Y432S, Y432A, Y432F, and deletion of Tyr 432 (Δ432). Also more preferably, the mutant SAH hydrolase is a derived sequence of amino acids set forth in SEQ ID NO:1 or encoded by the sequence of nucleotides set forth in SEQ ID NO:2 and has a combination of Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations. The nucleic acid molecules contemplated also include those that have conservative amino acid changes, and include those that hybridize along their full length to the coding portion of the sequence of nucleotides set forth in SEQ ID NO:2, under medium stringency, or preferably high stringency, such that the encoded protein retains ability to bind to the selected analyte without substantial conversion of the analyte.

Also provided herein is an isolated nucleic acid fragment, either DNA or RNA, that includes a sequence of nucleotides encoding a mutant S-adenosylhomocysteine (SAH) hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has enhanced binding affinity for homocysteine Hcy, SAH or adenosine but has attenuated catalytic activity.

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In one specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the attenuated catalytic activity is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof.

In another specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

In still another specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase irreversibly binds SAH.

In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase has a Km for SAH that is about or less than 10.0 μM. Preferably, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase has a Km for SAH that is about 1.0 μM or less than 1.0 μM.

In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase has a Kcat for SAH that is about or less than 0.1 S^{-1}.

In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase has one or more insertion, deletion, or point mutation(s). Preferably, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase is derived from a sequence of nucleotides set forth in SEQ ID NO:1 and has one or more mutation selected from R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S, L409G, S420T, P424A, F425S, P427A, D428G, H429A, Y430T, R431K, R431G, Y432S, Y432A, Y432F, and deletion of Tyr 432 (Δ432). Also more preferably, the isolated nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase is derived from a sequence of nucleotides set forth in SEQ ID NO:1 and has a combination of Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

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Further provided is a plasmid, including the nucleic acid fragment encoding the above mutant SAH hydrolases. Preferably, the plasmid is an expression vector including a sequence of nucleotides encoding: a) a promoter region; and b) a mutant S-adenosylhomocysteine (SAH) hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has enhanced

5   binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity. The sequence of nucleotides encoding the mutant SAH hydrolase is operatively linked to the promoter, whereby the mutant SAH hydrolase is expressed. More preferably, the plasmid also includes a selectable marker.

Further provided is a recombinant host cell containing the above plasmid. The recombinant

10   host cell can be any suitable host cell, including, but not limited to, a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell or an animal cell.

Also provided are methods for producing a mutant SAH hydrolase. The recombinant host cells can be grown or cultured under conditions whereby the mutant SAH hydrolase is expressed by the cell. The expressed mutant SAH hydrolase can then be isolated or recovered.

15   Additional mutant SAH hydrolase that substantially retains its binding affinity or has enhanced binding affinity for Hcy, SAH or adenosine, but has attenuated catalytic activity can be produced according to the procedures known to the those of skill in the art. The above-described mutant SAH hydrolases and additional mutant SAH hydrolase that substantially retain binding affinity or have enhanced binding affinity for Hcy, SAH or adenosine but have attenuated catalytic

20   activity can be used for assaying Hcy in a sample.

Hcy assays using mutant SAH hydrolase

In one specific embodiment, the mutant Hcy-binding enzyme used in the Hcy assay is a mutant SAH hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has

25   enhanced binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity. This assay, illustrated in detail in the EXAMPLES, is depicted in Figure 1. In this Figure, the homocysteine-containing analyte is reduced to produce Hcy, which, is quantified or detected by binding it to a mutant (substrate trapping) SAH hydrolase; the Hcy is then converted to SAH by reaction with adenosine in the presence of wild type SAH hydrolase. As exemplified in the Figure,

30   instead of using a monoclonal antibody to effect quantitation (see, *e.g.*, U.S. Patent No. 5,885,767

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and U.S. Patent No. 5,631,127). Quantitation is effected using a fluorescence-labeled tracer S-adenosylcysteine in a competition binding format in which the mutant SAH is used to trap the substrate. Any suitable quantitation assay with any suitable label can be used in the substrate trapping method. Figure 2 depicts an exemplary assay performed in a 96 well format; and figure 3

5   exemplifies preparation of labeling of adenosyl-cysteine with a fluorescent moiety.

In one preferred embodiment, the attenuated catalytic activity in the mutant SAH hydrolase is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof.

In another preferred embodiment, the mutant SAH hydrolase has attenuated 5'-hydrolytic

10   activity but substantially retains its 3'-oxidative activity.

In another preferred embodiment, the mutant SAH hydrolase irreversibly binds SAH.

In still another preferred embodiment, the mutant SAH hydrolase has a Km for SAH that is about or less than 10.0 μM. More preferably, the mutant SAH hydrolase has a Km for SAH that is about 1.0 μM or less than 1.0 μM.

15   In yet another preferred embodiment, the mutant SAH hydrolase has a Kcat for SAH that is about or less than 0.1 S^{-1}.

In yet another preferred embodiment, the mutant SAH hydrolase has one or more insertion, deletion, or point mutation(s). More preferably, the mutant SAH hydrolase is derived from the sequence of amino acids set forth in SEQ ID NO:1 or encoded by the sequence of nucleotides set

20   forth in SEQ ID No. 2 and has one or more of the following mutations: R38E, C53S, L54G, T57G, T57S, E59D, N80G, S83G, Y100T, K121A, D131E, D134E, E155G, T157G, T158Y, T159Y, N181D, N181A, D190A, N191A, L214A, Y221S, K226A, F235S, I240L, N248A, D263G, G269D, R285D, D292G, H301T, K309R, K322G, R329A, L347F, L347Y, L347I, M351A, H353R, S361G, F362S, Y379S, L386A, K388G, H398A, K401R, K401D, T407S, L409G, S420T,

25   P424A, F425S, P427A, D428G, H429A, Y430T, R431K, R431G, Y432S, Y432A, Y432F, and deletion of Tyr 432 (Δ432). Also more preferably, the mutant SAH hydrolase is derived from a sequence of amino acids set forth in SEQ ID NO:2 and has a combination of Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

In yet another preferred embodiment, prior to the contact between the sample and the

30   mutant SAH hydrolase, oxidized Hcy in the sample is converted into reduced Hcy. More

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preferably, the oxidized Hcy in the sample is converted into reduced Hcy by a reducing agent such as tri-n-butylphosphine (TBP), ß-ME, DTT, dithioerythritol, thioglycolic acid, glutathione, tris(2-carboxyethyl)phosphine, sodium cyanoborohydride, NaBH$_4$, KBH$_4$ and free metals.

In yet another preferred embodiment, prior to the contact between the sample and the mutant SAH hydrolase, the Hcy in the sample is converted into SAH. More preferably, the Hcy in the sample is converted into SAH by a wild-type SAH hydrolase. Also more preferably, the SAH is contacted with the mutant SAH hydrolase in the presence of a SAH hydrolase catalysis inhibitor such as neplanocin A or thimersol.

In yet another preferred embodiment, prior to the contact between the SAH and the mutant SAH hydrolase, free adenosine is removed or degraded. More preferably, the free adenosine is degraded by combined effect of adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase.

Any adenosine deaminase can be used. Preferably, the adenosine deaminase encoded by the nucleic acids having the following GenBank accession Nos. can be used: AF051275 (Caenorhabditis elegans); AI573492 (mouse mammary gland); AI462267 (mouse mammary gland); AI429519 (mouse embryo); AI429513 (mouse embryo); AI326688 (Mus musculus); AI324114 (mouse placenta); AI322477 (mouse placenta); AI152550 (mouse uterus); U76422 (Human, see also Lai, *et al.*, *Mol. Cell. Biol.*, 17(5):2413-24 (1997)); U76421 (Human); U76420 (Human); AI120695 (mouse mammary gland); AI049175 (Mus musculus); U73107 (Mus musculus); AF052506 (Mus musculus); AA871919 (Barstead bowel, Mus musculus); AA871917 (Barstead bowel, Mus musculus); AA871865 (Barstead bowel); AA871752 (Barstead bowel); AA871702 (Barstead bowel); AA871324 (Barstead bowel); AA871189 (Barstead bowel); AA869711 (Mus musculus); AA869187 (Mus musculus); AA869184 (Mus musculus); AA869176 (Mus musculus); AA869120 (Mus musculus); U75503 (Homo sapiens); AA646698 (mouse mammary gland); AA646681 (mouse mammary gland); AA427106 (mouse mammary gland); D50624 (Streptomyces virginiae); AA389303 (mouse embryo); AA389067 (mouse embryo); U88065 (Xenopus laevis); AA124740 (Mus musculus); U74586 (Rattus norvegicus); AA036487 (mouse placenta); AA035873 (mouse placenta); AA030290 (mouse placenta); AA023505 (mouse placenta); AA023331 (mouse placenta); AA111514 (mouse embryo); AA111327 (mouse embryo); AA110493 (mouse embryo); U73185 (Mus musculus); AA107590 (mouse embryo); AA102891

(mouse embryo); AA097525 (mouse embryo); AA096642 (mouse embryo); AA087094 (mouse embryo); AA060462 (mouse); U10439 (Human); M13792 (Human); U18942 (Rattus norvegicus); K02567 (Human); M10319 (Mouse); M59033 (*E. coli* adenosine). Preferably, the adenosine deaminase encoded by the nucleic acids having the following GenBank accession No. can be used:

5   U76422 (Human, see also Lai, *et al.*, *Mol. Cell. Biol.*, 17(5):2413-24 (1997)).

Any purine nucleoside phosphorylase can be used. Preferably, the purine nucleoside phosphorylase encoded by the nucleic acids having the following GenBank accession Nos. can be used: U88529 (E.coli); U24438 (E.coli, see also Cornell and Riscoe, *Biochim. Biophys. Acta*, 1396(1):8-14 (1998)); U83703 (H. pylori); and M30469 (*E. coli*).

10   Any xanthine oxidase can be used. Preferably, the xanthine oxidase encoded by the nucleic acids having the following GenBank accession Nos. can be used: AF080548 (Sinorhizobium meliloti); and U39487 (Human, see also Saksela and Raivio, *Biochem. J.*, 315(1):235-9 (1996)).

In yet another preferred embodiment, the sample containing or suspected of containing SAH is contacted with the mutant SAH hydrolase in the presence of a labeled SAH or a derivative

15   or an analog thereof, whereby the amount of the labeled SAH bound to the mutant SAH hydrolase inversely relates to amount of the SAH in the sample. The SAH, or the derivative or analog thereof, can be labeled by methods known in the art, *e.g.*, to become radioactive, enzymatic, fluorescent, luminescent (including chemo- or bio-luminescent) labeled. More preferably, the labeled SAH derivative or analog is a fluorescence labeled adenosyl-cysteine.

20   In yet another preferred embodiment, the sample containing or suspected of containing SAH is contacted with a labeled mutant SAH hydrolase. The mutant SAH hydrolase can be labeled by methods known in the art, *e.g.*, to become radioactive, enzymatic, fluorescent, luminescent (including chemo- or bio-luminescent) labeled. More preferably, the mutant SAH hydrolase is fluorescently labeled. For example, a mutant SAH hydrolase derived from an SAH

25   hydrolase having sequence of amino acids encoded by the sequence of nucleotides set forth in SEQ ID NO:2 is used and the mutant SAH hydrolase is fluorescently labeled at residue Cys421.

The present method can be used alone, or can be used in combination with other related assays. For example, the present method can further comprise a step of detecting cholesterol and/or folic acid in the sample, whether sequentially or simultaneously. Any suitable methods for

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assaying cholesterol and/or folic acid can be used. For example, the cholesterol and/or folic acid assays disclosed in WO 01/02600 can be used.

C.   Combinations, kits and articles of manufacture

In another aspect, the present invention is directed to a combination, which combination comprises: a) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) reagents for detecting binding between Hcy, SAH or adenosine and said SAH hydrolase. The combination can further comprise a reagent for detecting cholesterol and/or folic acid.

A kit comprising the above the combination is also provided. The kit can further comprise instructions for assaying Hcy, SAH or adenosine in a sample.

In still another aspect, the present invention is directed to an article of manufacture, which article of manufacture comprises: a) packaging material; b) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and c) a label indicating that the mutant SAH hydrolase and the means for use in assaying Hcy in a sample.

The packages discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems. Such packages include glass and plastic, such as polyethylene, polypropylene and polycarbonate, bottles and vials, plastic and plastic-foil laminated envelopes and the like. The packages may also include containers appropriate for use in auto analyzers. The packages typically include instructions for performing the assays.

D.   Conjugates

In yet another aspect, the present invention is directed to a conjugate, which conjugate comprises: a) a mutant SAH hydrolase that has binding affinity for Hcy, SAH or adenosine but has attenuated catalytic activity, wherein said binding affinity and/or said attenuated catalytic activity

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of said SAH hydrolase is caused by a mutation in said mutant SAH hydrolase's catalytic site, its

binding site for NAD^+, NADH, Hcy, SAH or adenosine, or a combination thereof; and b) a

facilitating agent linked to the mutant SAH hydrolase directly or via a linker, wherein the agent

facilitates: i) affinity isolation or purification of a conjugate; ii) attachment of a conjugate to a

5   surface; or iii) detection of a conjugate. The conjugate can be a fusion protein. Alternatively, the

mutant SAH hydrolase and the facilitating agent can be linked by other means.

The conjugates can be produced by chemical conjugation, such as via thiol linkages, but are

preferably produced by recombinant means as fusion proteins. In the fusion protein, the peptide or

fragment thereof is linked to either the N-terminus or C-terminus of the mutant enzyme. In

10   chemical conjugates the peptide or fragment thereof may be linked anywhere that conjugation can

be effected, and there may be a plurality of such peptides or fragments linked to a single mutant

enzyme or to a plurality thereof.

Conjugation can be effected by any method known to those of skill in the art. As described

below, conjugation can be effected by chemical means, through covalent, ionic or any other

15   suitable linkage. For example, the reagents and methods for conjugation as disclosed in WO

01/02600 can be used.

In some embodiments, the conjugate is a fusion protein, which prior to the contact between

the sample and the fusion protein, is isolated or purified. More preferably, the fusion protein is

isolated or purified through affinity binding between the protein or peptide fragment of the fusion

20   protein and an affinity binding moiety. Any kind of affinity interaction can be used for isolating or

purifying the fusion protein. The affinity interactions, such as those described herein, but not

limited to, are protein/protein, protein/nucleotide, protein/lipid, protein/polysaccharide, or

protein/metal interactions.

In other embodiments, prior to the contact between the sample and the conjugate, such as a

25   fusion protein, the conjugate is attached to a surface. More preferably, the conjugate is attached to

the surface through affinity binding between the facilitating agent of conjugate and an affinity

binding moiety on the surface. Any kind of affinity interaction can be used for attaching the

conjugate, including the protein/protein, protein/nucleotide, protein/lipid, protein/polysaccharide,

or protein/metal interactions.

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E.   Examples

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Preparation of mutant SAH hydrolase-encoding nucleic acid

Human placental SAH hydrolase gene (SEQ ID No. 1) was subcloned into an expression vector pKK223-3 (Pharmacia Biotech, Piscataway, New Jersey) at the EcoR I site. pKK223-3 contains the strong tac promoter upstream from the multiple cloning site and the strong rrnB ribosomal terminator downstream for control of protein expression. The SAH hydrolase gene-containing expression vector was transferred into an E. coli strain JM109 (Invitrogen, Carlsbad, CA). Site-directed mutagenesis of SAH hydrolase was conducted in two ways: 1) single-strand DNA-based M13 method; and 2) double-strand DNA-based PCR method.

Single-strand DNA-based mutagenesis

Single-strand DNA-based mutagenesis was conducted based on the method described by Taylor, et al., *Nucleic Acids Res.*, 13:8765-8785 (1985), which exploits the inability of NciI to cleave a thio-containing DNA strand. Sculptor™ invitro mutagenesis system RPN1526 (Amersham Life science, UK) was used. The pKK223-3 vector containing the wild type gene of SAH hydrolase was prepared using the method of alkaline lysis followed by plasmid purification using Promega's DNA purification kit (Wizard plus Minipreps, Promega, Madison WI). The purified plasmid was digested with EcoR I (Stratagene, La Jolla, CA) at 37°C for 2 hours to obtain the EcoR I fragment by agarose gel electrophoresis followed by DNA purification using Promega DNA purification kit. The purified EcoR I fragment was subcloned into M13 mp19 DNA (Pharmacia Biotech, Piscataway, New Jersey) by T4 DNA ligase (Pharmacia Biotech Piscataway, New Jersey). The ligation was conducted in One-phor-All buffer (10 mM tris-Ac, pH 7.5, 10 mM Mg(Ac)2, 50 mM KAc; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) at 4°C overnight. The ligation product was transferred into TG1 cells (Stratagene, La Jolla, CA) by incubation of 10 μl of the M13 with 90 μl of competent TG 1 cells at 0°C for 30 min. and 42°C for 75 sec. After

being chilled to 0°C for 2 min, 500 μl of 2XYT media was added to the cells and incubated for 10 min. at 37°C. Two hundred μl of growing nontransformed TG1 cells were mixed with the transformed TG1 cells, and to which 2.5 ml of soft agarose LB (42°C) was added. The cell mixture was immediately poured onto preheated LB agar plates (40°C), and incubated at 37°C

5   overnight. Phage clones were picked up for examination of the insertion of SAH hydrolase gene and the orientation through DNA sequencing and restriction enzyme analysis. The selected phage clone was used for preparation of single strand DNA template.

The M13 phage containing the SAH hydrolase gene were incubated with TG1 cells in 3 ml of 2xYT media overnight. One drop of the overnight culture was mixed with growing TG1 cells

10   (in log phase) in 30 ml of 2XYT media. Cells were incubated for 8 hours with shaking. After centrifugation, the supernatant was collected for single-strand template DNA purification. The purification was conducted according to the manufacture's procedure provided by Amersham Life Science.

Design of primers for point mutation

15   Oligonucleotides (15-30 bases) were synthesized by CruaChem (Sterling, VA). The sequence of the oligonucleotides were designed to be complementary to the sequence in the region covering both sides of the mutation site. For example, to mutate lys 426 to glu 426, the oligonucleotides used as primer contained the following sequence: GGCCCCTTC**GAG**CCGGATCACTACCGC (SEQ ID NO:4) where **GAG** codes for glu instead

20   of original (wild type) **AAG** which codes for lys.

The selection of mutation sites was based on x-ray structure of the substrate binding site and coenzyme binding site of human SAH hydrolase (Turner, *et al.*, *Nature Structural Biology*, 5:369-376 (1998)). Amino acid residues such as Thr 157, Asp 131, Hys 301, Lys 186, Asn 191, Glu 156, Asp 190, Phe 362, Phe 302, Asn 181, His 353, Glu 59, Ser 83, His 55, Leu 54, Cys 79,

25   His 301, Arg 343, Asp 303, Leu 344, Asn 80, Asn 346, Asp 107 and entire C-terminal residues can be the mutagenesis targets (see Table 2 for particular mutations generated). The coenzyme binding domain contains residues from Tyr193-Asn346.

The oligonucleotides were dissolved in water to a concentration of 5 ng/μl. The oligonucleotide solution was then phosphorylated at the 5'-end using polynucleotide kinase. The

phosphorylation reaction mixture contained the following materials: 2.5 μl of oligonucleotides (5 ng/μl), 3 μl of one-phor-all 10X kinase buffer (Pharmacia Biotech), 21.5 μl of water, 2 μl of 10 mM ATP, and 1 μl of polynucleotide kinase (100,000U/ml) (Pharmacia Biotech). The reaction mixture was incubated at 37°C for 30 min. followed by heating at 70°C for 10 min. to inactivate

5   the enzyme.

Table 1.   Oligonucleotides used for site-directed mutagenesis of human SAH hydrolases

Mutant	Sequence	SEQ ID No.	Fo/Re
K186A	GACTTCGTCACC**GCC**AGCAAGTTTGGG	5	Fo
K186A	CCCAAACTTGCT**GGC**GGTGACGAAGTC	6	Re
F302S	AACATTGGACAC**TCT**GACGTGGAGATC	7	Fo
F302S	GATCTCCACGTC**AGA**GTGTCCAATGTT	8	Re
H301D	TGTAACATTGGA**GAC**TTTGACGTGGAG	9	Fo
H301D	CTCCACGTCAAA**GTC**TCCAATGTTACA	10	Re
H353S	TGTGCCATGGGC**TCC**CCCAGCTTCGTG	11	Fo
H353S	CACGAAGCTGGGG**GA**GCCCATGGCACA	12	Re
R343A	CTGGCCGAGGGTG**CG**CTGGTCAACCTG	13	Fo
R343A	CAGGTTGACCAGC**GC**ACCCTCGGCCAG	14	Re
D190A	AAGAGCAAGTTT**GCC**AACCTCTATGGC	15	Fo
D190A	GCCATAGAGGTT**GGC**AAACTTGCTCTT	16	Re
F82A	AGCTGCAACATC**GCC**TCCACCCAGGAC	17	Fo
F82A	GTCCTGGGTGGA**GGC**GATGTTGCAGCT	18	Re
N181D	GCCATCAATGTC**GAC**GACTCCGTCACC	19	Fo
N181D	GGTGACGGAGTC**GTC**GACATTGATGGC	20	Re
R431A	CCGGATCACTAC**GCC**TACTGAGAATTC	21	Fo
R431A	GAATTCTCAGTA**GGC**GTAGTGATCCGG	22	Re
K426R	GATGGCCCCTTC**CGC**CCGGATCACTAC	23	Fo
K426R	GTAGTGATCCGG**GCG**GAAGCCATCACA	24	Re
C195S	AACCTCTATGGC**TCC**CGGGAGTCCCTC	25	Fo
C195S	GAGGGACTCCCGG**GA**GCCATAGAGGTT	26	Re
Δ432	GATCACTACCGC**TGA**TGAGAATTCGAG	27	Fo
Δ432	CTCGAATTCTCA**TCA**GCGGTAGTGATC	28	Re

The mutagenized codon is underlined, and the nucleotides changed are in boldface type.
10   Fo: forward oligonucleotide
    Ro: backward oligonucleotide

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Table 2. Additional oligonucleotides used for site-directed mutagenesis of human SAH hydrolases

Mutant	Sequence	SEQ ID No.	Fo/Re
Glu156Ala	GGCATCTCTGAGGCGACCACGACTGGG	29	Fo
Glu156Ala	CCCAGTCGTGGTCGCCTCAGAGATGCC	30	Re
Glu156Asp	GGCATCTCTGAGGACACCACGACTGGG	31	Fo
Glu156Asp	CCCAGTCGTGGTGTCCTCAGAGATGCC	32	Re
Asp131Lys	CTCAACATGATTCTGGACAAGGGGGGCGACCTCACC	33	Fo
Asp131Lys	GGTGAGGTCGCCCCCCTTGTCCAGAATCATGTTGAG	34	Re
Asp131Asn	CTCAACATGATTCTGGACAACGGGGGCGACCTCACC	35	Fo
Asp131Asn	GGTGAGGTCGCCCCCGTTGTCCAGAATCATGTTGAG	36	Re
Lys186Ala	GACTCCGTCACCGCGAGCAAGTTTGAC	37	Fo
Lys186Ala	GTCAAACTTGCTCGCGGTGACGGAGTC	38	Re
Lys186Asp	GACTCCGTCACCGACAGCAAGTTTGAC	39	Fo
Lys186Asp	GTCAAACTTGCTGTCGGTGACGGAGTC	40	Re
His55Pro	GCTGGCTGCCTGCCCATGACCGTGGAGACG	41	Fo
His55Pro	CGTCTCCACGGTCATGGGCAGGCAGCCAGC	42	Re
Arg343Ala	CTGCTGGCCGAGGGTGCGCTGGTCAACCTG	43	Fo
Arg343Ala	CAGGTTGACCAGCGCACCCTCGGCCAGCAG	44	Re
Asp303Glu	GTGTGTAACATTGGACACTTTGAGGTGGAGATCGATGTC	45	Fo
Asp303Glu	GACATCGATCTCCACCTCAAAGTGTCCAATGTTACACAC	46	Re
Phe302Ile	GTGTGTAACATTGGACACATTGACGTGGAGATC	47	Fo
Phe302Ile	GATCTCCACGTCAATGTGTCCAATGTTACACAC	48	Re
Leu344Gly	GCCGAGGGTCGGGGGGTCAACCTGGGTTGTGCC	49	Fo
Leu344Gly	GGCACAACCCAGGTTGACCCCCCGACCCTCGGC	50	Re
Phe82Ser	CAGTGGTCCAGCTGCAACATCTCCTCCACCCAGGAC	51	Fo
Phe82Ser	GTCCTGGGTGGAGGAGATGTTGCAGCTGGACCACTG	52	Re
Thr159Ser	GAGGAGACCACGTCCGGGGTCCACAACCTC	53	Fo
Thr159Ser	GAGGTTGTGGACCCCGGACGTGGTCTCCTC	54	Re
Asn346Gly	GGTCGGCTGGTCGGCCTGGGTTGTGCC	55	Fo
Asn346Gly	GGCACAACCCAGGCCGACCAGCCGACC	56	Re
Asn346Asp	GGTCGGCTGGTCGACCTGGGTTGTGCC	57	Fo
Asn346Asp	GGCACAACCCAGGTCGACCAGCCGACC	58	Re
Cys79Ala	GTGCAGTGGTCCAGCGCCAACATCTTCTCCACC	59	Ro
Cys79Ala	GGTGGAGAAGATGTTGGCGCTGGACCACTGCAC	60	Re
Cys79Gly	GTGCAGTGGTCCAGCGGCAACATCTTCTCCACC	61	Fo
Cys79Gly	GGTGGAGAAGATGTTGCCGCTGGACCACTGCAC	62	Re
His301Ala	GTGTGTAACATTGGAGCCTTTGACGTGGAG	63	Fo
His301Ala	CTCCACGTCAAAGGCTCCAATGTTACACAC	64	Re
Asp303Ala	GTGTGTAACATTGGACACTTTGCCGTGGAGATCGATGTC	65	Fo
Asp303Ala	GACATCGATCTCCACGGCAAAGTGTCCAATGTTACACAC	66	Re
Thr158Tyr	ATCTCTGAGGAGACCTATACTGGGGTCCACAAC	67	Fo
Thr158Tyr	GTTGTGGACCCCAGTATAGGTCTCCTCAGAGAT	68	Re

Mutant	Sequence	SEQ ID No.	Fo/Re
Thr159Tyr	ATCTCTGAGGAGACCACGTATGGGGTCCACAAC	69	Fo
Thr159Tyr	GTTGTGGACCCCATACGTGGTCTCCTCAGAGAT	70	Re

Fo: forward oligonucleotide
Ro: backward oligonucleotide.

Table 3.   Additional oligonucleotides used for site-directed mutagenesis of human SAH hydrolases

Mutant	Sequence	SEQ ID No.	Fo/Re
L347F	GGTCGGCTGGTCAACTTTGGTTGTGCCATG	71	Fo
L347F	CATGGCACAACCAAAGTTGACCAGCCGACC	72	Re
R38E	ATGCGTATGCGGGAGGAATACTCGGCCTCC	73	Fo
R38E	GGAGGCCGAGTATTCCTCCCGCATACGCAT	74	Re
C53S	GCCCGCATCGCTGGCTCTCTGCACATGACC	75	Fo
C53S	GGTCATGTGCAGAGAGCCAGCGATGCGGGC	76	Re
T57G	GGCTGCCTGCACATGGGGGTGGAGACGGCC	77	Fo
T57G	GGCCGTCTCCACCCCCATGTGCAGGCAGCC	78	Re
E59D	CTGCACATGACCGTGGATACGGCCGTCCTC	79	Fo
E59D	GAGGACGGCCGTATCCACGGTCATGTGCAG	80	Re
T57S	GGCTGCCTGCACATGTCTGTGGAGACGGCC	81	Fo
T57S	GGCCGTCTCCACAGACATGTGCAGGCAGCC	82	Re
L347Y	GGTCGGCTGGTCAACTATGGTTGTGCCATG	83	Fo
L347Y	CATGGCACAACCATAGTTGACCAGCCGACC	84	Re
L347I	GGTCGGCTGGTCAACATTGGTTGTGCCATG	85	Fo
L347I	CATGGCACAACCAATGTTGACCAGCCGACC	86	Re
S83G	TGCAACATCTTCGGTACCCAGGACCATGCG	87	Fo
S83G	TGCAACATCTTCGGTACCCAGGACCATGCG	88	Re
L54G	GCCCGCATCGCTGGCTGCGGTCACATGACC	89	Fo
L54G	GGTCATGTGACCGCAGCCAGCGATGCGGGC	90	Re
Y100T	GGCATTCCGGTGACTGCCTGGAAGGGCGAA	91	Fo
Y100T	TTCGCCCTTCCAGGCAGTCACCGGAATGCC	92	Re
K121A	ACCCTGTACTTCGCTGACGGGCCCCTCAAC	93	Fo
K121A	GTTGAGGGGCCCGTCAGCGAAGTACAGGGT	94	Re
N191A	AGCAAGTTTGACGCTCTCTATGGCTGCCGG	95	Fo
N191A	CCGGCAGCCATAGAGAGCGTCAAACTTGCT	96	Re
M351A	CTGGGTTGTGCCGCTGGCCACCCCAGCTTC	97	Fo
M351A	GAAGCTGGGGTGGCCAGCGGCACAACCCAG	98	Re
H353R	CTGGGTTGTGCCATGGGCCGTCCCAGCTTC	99	Fo
H353R	GAAGCTGGGACGGCCCATGGCACAACCCAG	100	Re
F362S	TTCGTGATGAGTAACTCCAGTACCAACCAG	101	Fo
F362S	CTGGTTGGTACTGGAGTTACTCATCACGAA	102	Re
D131E	ATGATTCTGGACGAAGGGGGCGACCTCACC	103	Fo

D131E	GGTGAGGTCGCCCCCTTCGTCCAGAATCAT	104	Re
T157G	ATCTCTGAGGAGGGTACGACTGGGGTCCAC	105	Fo
T157G	GTGGACCCCAGTCGTACCCTCCTCAGAGAT	106	Re
N80G	TGGTCCAGCTGCGGTATCTTCTCCACCCAG	107	Fo
N80G	CTGGGTGGAGAAGATACCGCAGCTGGACCA	108	Re
D134E	ATGATTCTGGACGACGGGGGCGAACTCACC	109	Fo
D134E	GGTGAGTTCGCCCCCGTCGTCCAGAATCAT	110	Re
E155G	ATCTCTGGTGAGACCACGACTGGGGTCCAC	111	Fo
E155G	GTGGACCCCAGTCGTGGTCTCACCAGAGAT	112	Re
N181A	GCCATCAATGTCGCTGACTCCGTCACCAAG	113	Fo
N181A	CTTGGTGACGGAGTCAGCGACATTGATGGC	114	Re
L214A	ATGATTGCCGGCGCTGTAGCGGTGGTAGCA	115	Fo
L214A	TGCTACCACCGCTACAGCGCCGGCAATCAT	116	Re
Y221S	GTGGTAGCAGGCTCTGGTGATGTGGGCAAG	117	Fo
Y221S	CTTGCCCACATCACCAGAGCCTGCTACCAC	118	Re
K226A	GGTGATGTGGGCGCTGGCTGTGCCCAGGCC	119	Fo
K226A	GGCCTGGGCACAGCCAGCGCCCACATCACC	120	Re
F235S	GCCCTGCGGGGGTTCTGGAGCCCGCGTCATC	121	Fo
F235S	GATGACGCGGGCTCCAGAACCCCGCAGGGC	122	Re
I240L	GGAGC CGCGTCCTTATCACCGAGATTGAC	123	Fo
I240L	GTCAATCTCGGTGATAAGGACGCGGGCTCC	124	Re
N248A	ATTGACCCCATCGCTGCACTGCAGGCTGCC	125	Fo
N248A	GGCAGCCTGCAGTGCAGCGATGGGGTCAAT	126	Re
D263G	GTGACCACCATGGGTGAGGCCTGTCAGGAG	127	Fo
D263G	CTCCTGACAGGCCTCACCCATGGTGGTCAC	128	Re
G269D	GAGGCCTGTCAGGAGGATAACATCTTTGTC	129	Fo
G269D	GACAAAGATGTTATCCTCCTGACAGGCCTC	130	Re
R285D	GACATCATCCTTGGCGATCACTTTGAGCAG	131	Fo
R285D	CTGCTCAAAGTGATCGCCAAGGATGATGTC	132	Re
D292G	CAGATGAAGGGTGATGCCATTGTGTGTAAC	133	Fo
D292G	GTTACACACAATGGCATCACCCTTCATCTG	134	Re
H301T	AACATTGGAACTTTTGACGTGGAGATCGAT	135	Fo
H301T	ATCGATCTCCACGTCAAAAGTTCCAATGTT	136	Re
K309R	GAGATCGATGTCAGATGGCTCAACGAGAAC	137	Fo
K309R	GTTCTCGTTGAGCCATCTGACATCGATCTC	138	Re
K322G	GTGAACATCGGTCCGCAGGTGGACCGGTAT	139	Fo
K322G	ATACCGGTCCACCTGCGGACCGATGTTCAC	140	Re
R329A	GACCGGTATGCTTTGAAGAATGGGCGCCGC	141	Fo
R329A	GCGGCGCCCATTCTTCAAAGCATACCGGTC	142	Re
S361G	ATGAGTAACGGTTTCACCAACCAGGTGATG	143	Fo
S361G	CATCACCTGGTTGGTGAAACCGTTACTCAT	144	Re
Y379S	CATCCAGACAAGTCTCCCGTTGGGGTTCAT	145	Fo
Y379S	ATGAACCCCAACGGGAGACTTGTCTGGATG	146	Re
L386A	GGGGTTCATTTCGCTCCCAAGAAGCTGGAT	147	Fo

L386A	ATCCAGCTTCTTGGGAGCGAAATGAACCCC	148	Re
K388G	CATTTCCTGCCCGGTAAGCTGGATGAGGCA	149	Fo
K388G	TGCCTCATCCAGCTTACCGGGCAGGAAATG	150	Re
H398A	GCAGTGGCTGAAGCCGCTCTGGGCAAGCTG	151	Fo
H398A	CAGCTTGCCCAGAGCGGCTTCAGCCACTGC	152	Re
K401R	CACCTGGGCCGTCTGAATGTGAAGTTGACC	153	Fo
K401R	GGTCAACTTCACATTCAGACGGCCCAGGTG	154	Re
K401D	CACCTGGGCGATCTGAATGTGAAGTTGACC	155	Fo
K401D	GGTCAACTTCACATTCAGATCGCCCAGGTG	156	Re
T407S	AATGTGAAGTTGTCTAAGCTAACTGAGAAG	157	Fo
T407S	CTTCTCAGTTAGCTTAGACAACTTCACATT	158	Re
L409G	GTGAAGTTGACCAAGGGTACTGAGAAGCAA	159	Fo
L409G	TTGCTTCTCAGTACCCTTGGTCAACTTCAC	160	Re
S420T	TACCTGGGCATGACTTGTGATGGCCCCTTC	161	Fo
S420T	GAAGGGGCCATCACAAGTCATGCCCAGGTA	162	Re
P424A	TCCTGTGATGGCGCTTTCAAGCCGGATCAC	163	Fo
P424A	GTGATCCGGCTTGAAAGCGCCATCACAGGA	164	Re
F425S	TGTGATGGCCCCTCTAAGCCGGATCACTAC	165	Fo
F425S	GTAGTGATCCGGCTTAGAGGGGCCATCACA	166	Re
D428G	TGTGATGGCCCCTTCAAGCCGGGTCACTAC	167	Fo
D428G	GTAGTGACCCGGCTTGAAGGGGCCATCACA	168	Re
P427A	TGTGATGGCCCCTTCAAGGCTGATCACTAC	169	Fo
P427A	GTAGTGATCAGCCTTGAAGGGGCCATCACA	170	Re
H429A	TGTGATGGCCCCTTCAAGCCGGATGCTTAC	171	Fo
H429A	GTAAGCATCCGGCTTGAAGGGGCCATCACA	172	Re
Y430T	GGCCCCTTCAAGCCGGATCACACTCGCTAC	173	Fo
Y430T	GTAGCGAGTGTGATCCGGCTTGAAGGGGCC	174	Re
R431K	GGCCCCTTCAAGCCGGATCACTACAAATAC	175	Fo
R431K	GTATTTGTAGTGATCCGGCTTGAAGGGGCC	176	Re
R431G	GGCCCCTTCAAGCCGGATCACTACGGTTAC	177	Fo
R431G	GTAACCGTAGTGATCCGGCTTGAAGGGGCC	178	Re
Y432S	CCCTTCAAGCCGGATCACTACCGCTCTTGA	179	Fo
Y432S	TCAAGAGCGGTAGTGATCCGGCTTGAAGGG	180	Re
Y432A	CCCTTCAAGCCGGATCACTACCGCGCTTGA	181	Fo
Y432A	TCAAGCGCGGTAGTGATCCGGCTTGAAGGG	182	Re
Y432F	CCCTTCAAGCCGGATCACTACCGCTTTTGA	183	Fo
Y432F	TCAAAAGCGGTAGTGATCCGGCTTGAAGGG	184	Re

Fo: forward oligonucleotide
Re: backward oligonucleotide

The 5'-phosphorylated oligonucleotides DNA was annealed with single-stranded DNA

5   (M13 phage containing wild type human SAH hydrolase gene, $1\mu g/\mu l$) in a ratio of

oligonucleotide: template of 2:1 in annealing buffer. The annealing reaction was performed by

35

incubating the annealing mixture at 70°C for 3 min. followed by 30 min. at 37°C or followed by

transferring the micro centrifuge tube to a 55°C beaker and then allowed to cool to room

temperature. To the annealing mixture (17 μl), 19 μl of dNTP A (α-S) mix, 1.5 μl of T7 DNA

polymerase (0.8 units), and 2.5 μl of T4 DNA ligase (92.5 units), and 6 μl of water were added.

5   After 10 min. at room temperature and 30 min. at 37°C, the reaction was stopped by heat

inactivation at 70°C for 15 min. To the reaction mixture was added T5 exonuclease (2000 units)

and exonuclease buffer to remove single-strand non-mutant DNA at 37°C for 30 min. followed by

15 min. of heat inactivation at 70°C. NciI (5 units) was added to the reaction mixture to nicking

the non-mutant strand by incubating NciI at 37°C for 90 min. The non-mutant strand was digested

10   by adding 160 units of Exonuclease III and incubating at 37°C for 30 min. followed by heat

inactivation. To repolymerize the gaped DNA, dNTP mix B and 3.5 units of DNA polymerase I

and 2.5 units of T4 DNA ligase were added to the reaction mixture, and incubated at 37°C for 1 h.

The M13 plasmid containing the mutated SAH hydrolase gene was then transferred into

competent TG 1 host cells by heat shock method or an electroporation method. Ten μl of the

15   mutant M13 plasmid was added to 90 μl of water and mixed with competent TG1 cells in ice for

40 min. The TG1 cells were shocked by incubation at 42°C for 45 sec. and immediately at 0°C for

5 min. The transferred TG1 cells were allowed to return to room temperature, and mixed with 200

μl of growing non-transferred TG1 cells (served as lawn cells). Three ml of molten Htop agar was

added and mixed followed by immediately pouring the cells onto a L plate. The plate was

20   incubated in 37°C for overnight. Phage plaques formed were picked by sterile tooth pick and

swirling in a tube containing 3 ml of 2XYT medium. After overnight culture, cells were collected

by centrifugation, and the double-strand M13 plasmid from the cells was purified by using

Promega DNA purification kit (Wizard plus Minipreps).

The supernatant from centrifugation was used to purify single-strand M13 DNA. The

25   mutation was confirmed by DNA sequencing of the single-strand M13 DNA using Sequenase

Version 2.0 (Unites States Biochemical). The double-strand M13 DNA containing correct

mutation sequence was selected, and digested with EcoR I. The EcoR I fragment containing the

mutant SAH hydrolase gene was purified by agarose electrophoresis followed by gene cleaning

using Qlaquick Gel Extraction kit (Qiagen, Valencia, CA). The purified EcoR I fragment was

subcloned into pKK223-3 expression vector using T4 ligase. Two μl of EcoR 1 treated and 5'-dephosphorylated pKK223-3 vector backbone was incubated with 10 μl of the purified mutant insert DNA in a backbone to insert ratio of 2:1. The ligation reaction was carried out in One-phore-All buffer containing 0.01 M ATP at 16C overnight. The ligated vector containing mutant

5   SAH hydrolase gene was transferred into competent *E. Coli* JM109 cells by heat shock method. The transformed cells were selected against 100 μl/ml ampicillin. Ampicillin-resistant clones were picked and grown in 10 ml of 2xYT medium containing 35 μl/ml ampicillin for 2 hours at 37°C and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and grown overnight at 37°C. The cells were harvested by centrifugation, and suspended in 0.8 ml of 50 mM Tri-HCl,

10   pH 7.5, containing 2 mM EDTA. Cells were lysed by rapid freezing and thawing. After centrifugation at 13,500 rpm for 1 hour at 4°C, the supernatant was collected for SDS-PAGE analysis for over-expression of SAH hydrolase mutant protein. A heavy protein band at molecular size of 47,000 Da indicates the overexpression of mutant SAH hydrolase protein.

PCR-based mutagenesis method

15   PCR-based mutagenesis was performed using the ExSite PCR-based Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The ExSite method uses increased template concentration and <10 PCR cycles. The resulting mixture of template DNA, newly synthesized DNA and hybrid parental/newly synthesized DNA is treated with Dpn I and Pfu DNA polymerase. Dpn I digests the *in vivo* methylated parental template and hybrid DNA, and Pfu DNA polymerase

20   polishes the ends to create a blunt-ended PCR product. The end-polished PCR product is then intramolecularly ligated together and transformed into *E. coli* cells. The detailed experimental procedure is described as follows:

To a microcentrifuge tube were added 0.5 pmol of template DNA, 2.5 μl of 10x mutagenesis buffers, 1μl of 25 mM dNTP mix, 15 pmol of each primer, and ddH$_2$O to a final

25   volume of 24 μl. To the reaction mixture was then added 1 μl of ExSite DNA polymerase blend (5 U/μl). The reaction solution was overlayed with 20 μl of mineral oil and thermal cycle the DNA using 7012 amplification cycles. The cycling parameters are listed in Table 10.

Table 3. Mutagenesis Cycling Parameters

Segment	Cycles	Temperature	Time
1	1	94°C	4 min.
		50°C	2 min.
		72°C	2 min.
2	8	94°C	1 min.
		56°C	2 min.
		72°C	1 min.
		72°C	5 min.
3		72°C	5 min.

Following amplification, the reaction tube was placed on ice for 2 min. to cool the reaction

to <37°C. To the reaction tube were added 1 μl of the Dpn I restriction enzyme (10 U/μl) and 0.5

5   μl of cloned Pfu DNA polymerase (2.5 U/μl) followed by incubation at 37°C for 30 min. The

reaction was stopped by heating at 72°C for 30 min. For ligating the product, to the reaction tube

were added 100 μl of ddH$_2$O, 10 μl of 10x mutagenesis buffer, and 5 μl of 10 mM rATP. Transfer

10 μl of the above reaction mixture to a new micocentrifuge tube and add 1 μl of T4 DNA ligase (4

U/μl). The ligation was incubated at 37°C for 1 hour. 2 μl of the ligated DNA was added to 80 μl

10   of Epicurian Coli XL1-Blue supercompetent cells on ice and incubated for 30 min. followed by 45

seconds at 42defendant and 2 min. on ice. The transformed cells were immediately plated on LB-

ampicillin agar plates which had been spread with 20 μl of 10% X-gal prepared in DMF and 20 μl

of 100 M IPTG in H$_2$O. The plate was incubated overnight at 37°C. The blue colonies were

selected as colonies containing the mutagenized plasmid. The selected colonies were further

15   confirmed by DNA sequencing. Protein overexpression and substrate trapping screening were

performed as described above.

Double-strand pKK223-3 containing human SAH hydrolase (wild type) was purified from

50 ml of *E. coli* JM109 culture using Promega DNA purification kit (Wizard plus Minipreps). The

purified plasmid was annealed with PCR primers containing the desired mutation sequence.

20   Deletion and insertion mutations were also performed according to the manufacture's

protocol using ExSite PCR-based Site-directed Mutagenesis Kit. Double mutations or

combinations of mutation and deletion or insertion were carried out using mutated or deleted DNA

as template for secondary mutation or deletion using either M13-based mutagenesis or PCR-based mutagenesis methods.

Identification of substrate trapping SAH hydrolase

The cell-free extracts from colonies that inducibly overexpressed mutant SAH hydrolase proteins were chromatographed on a monoQ column (HR5/5) equipped with FPLC system. Proteins were eluted with a linear gradient of NaCl from 0 to 1 M in 10 mM sodium phosphate buffer, pH 7.0 over 35 min. The major protein peak that eluted at the same or close retention time as that of the wild type SAH hydrolase was collected. An aliquot collected mutant SAH hydrolase (1-10 μg) was incubated with [^{3}H]SAH (10 mCi/mmole, 200 μM) and 30 μM of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) at room temperature for 5-30 min.

The reaction solution was filtered through a membrane of molecular weight cut-off at 30,000 by centrifugation. The filtrate was measured at 412 nm for Hcy formation (enzyme activity) and the [^{3}H] radioactivity on the membrane was measured by scintillation counting after membrane washing with 1 ml of 50 mM phosphate buffer, pH 7.0.

The mutant hydrolases that show high radioactivity on the membrane and low O.D. at 412 nm of the filtrate relative to the wild type enzyme were selected as candidates for further characterization including determination of Km or Kd and binding energy (ΔG). Mutant SAH hydrolases with Km value lower than 10 μM toward SAH and kcat value lower than 0.1 per second were overexpressed in larger quantity (1-2 L of *E. coli* culture) and the enzyme proteins were purified to homogenous as judged by single band on SDA-PAGE.

EXAMPLE 2

Large scale overexpression and purification of wild type and mutant forms of SAH hydrolases

Purification

The cell-free extract of IPTG-induced *E. Coli* JM109 (containing SAH hydrolase gene in pKK223-3 vector) culture was mixed with powder DEAE-cellulose (Sigma, St. Louis, MO) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mM EDTA (buffer A). The cell-free extract and DEAC-cellulose mixture was placed in a funnel and filtrated under vacuum. After washing with 3 volumes of buffer A, the filtrate was precipitated by solid ammonium sulfate

(30-60%). The precipitated protein was collected by centrifugation at 13000 rpm, and resuspended in 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA. The protein was chromatographed through a Sephacryl S-300 size exclusion column (2.5X100 cm) (Pharmacial Biotech, Piscataway, New Jersey) followed by a DEAE-Sepharose ion exchange column (2.5X30 cm) eluted by a linear NaCl gradient. The major protein peak from DEAE-Sepharose was examined by SDS-PAGE. In most of the times, this purification procedure gave a single protein band on SDS-PAGE. Sometime, minor bands were observed on SDS-PAGE. In this case, rechromatography on DEAE-Sepharose column was performed to obtain pure protein. SAH hydrolase activity or [^{3}H]SAH binding affinity was also measured to confirm the protein peak.

Storage of the purified SAH hydrolase

The purified wild type and mutant SAH hydrolases were dialyzed against 5 mM sodium phosphate buffer, pH 7.0 for 6 hours at 4°C. The protein was then frozen in liquid nitrogen and lyophilized under vacuum. The lyophilized protein was stored at -70$^{\circ C}$. The protein was stable for at least 2 years. The purified protein can also be stored in liquid containing 20% of glycerol at -20°C. For wild type enzyme, addition of 5 mole excess of adenosine (Ado) to the 20% glycerol solution stabilizes the enzyme activity even better.

Assays for enzyme activity

The assay of SAH hydrolase activity in the hydrolytic direction was performed as described in Yuan, *et al., J. Biol. Chem.*, **271**:28008-28016, 1996). The assay measures the hydrolysis of SAH into Ado and Hcy. The reaction product Hcy was derivatized by thiol specific reagent DTNB for colometric determination at 412 nm. The assay for SAH hydrolase in the synthetic direction was measured by the formation of SAH from substrate Ado and Hcy using HPLC (see, Yuan, *et al., J. Biol. Chem.*, **268**:17030-17037 (1993). One unit of the enzyme activity was defined as the amount of enzyme that can hydrolyze or synthesize 1 μ mole of SAH/min/mg.

Assay for binding affinity (Kd)

For mutant enzyme that completely lacks activity, the binding constant (Kd) values were determined by an equilibrium dialysis technique using [^{3}H] SAH and Spectrum 5-cell Equilibrium

Dialyzer) (Spectrum, Houston, Texas). The membrane disc used had molecular cut-off of 25,000. Kd values for several exemplary SAH hydrolase mutant enzymes are shown in Figure 5.

<div align="center">EXAMPLE 3</div>

Preparation of reagents

5   **Preparation of fluorophore-labeled Ado and SAH analogs**

Method 1

Ado-5'-carboxylic acid (Sigma, St. Louis, MO) was derivatized with 9-(hydroxylmethyl)anthracene (HMA) (Fluka, Buchs, Switzerland). To 10 mg of Ado-5'-carboxylic acid dissolved in 100 ml of chloroform (10 min sonication) was added 50 mg 1-

10   hydroxybenzotriazole (HOBT) (Janssen Chimica, Beerse, Belgium). After evaporation to dryness under nitrogen, 300 mg of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride in 300 ml chloroform and 5 ml of triethylamine were added. The resulting solution was kept at 0°C for 30 min. To the above reaction mixture was added 200 mg HMA in 100 ml of chloroform. The mixture was allowed to stand at room temperature for 10 min. and then evaporated to dryness

15   under a stream of nitrogen. The residue obtained was dissolved in 10 ml of HPLC mobile phase (methanol-water mixture, 90:10, w/w). One ml of the above solution was injected into a semi-preparative column (Econosphere, C18, 7x300 mm, Alltech, Dearfield, IL). The column was eluted with an isocratic method. The flow rate was 2 ml/min. The peaks were monitored at UV260 nm and fluorescence at Ex-365nm, Em-415nm. The peaks with UV and fluorescence

20   absorbance were collected as HMA-labeled Ado-5'-ester.

Method 2

Ado-5'caroboxylic acid and 4-bromomethyl-7-methoxycoumarin (Br-Mmc) (Sigma, St. Louis, MO) were dissolved in ethyl acetate in a molar ratio of 1:3. The reaction volume was 25 ml. After addition of 2 g of finely powdered K_2CO_3 the solution was refluxed for 1 hour using a

25   ml-reluxer. After cooling, the reaction solution was injected into a C18 column (Econosphere, C18, 7x300 mm, Alltech, Deerfield, IL) for HPLC separation. The elution was monitored by UV

<div align="center">41</div>

(260 nm) and fluorescence (Em 328nm and Ex390nm). The elution was performed in a linear gradient of methanol:water from 20 to 100% over 30 min. The flow rate was 2 ml/min.

Method 3

This method is depicted in Figure 3. Adenosyl-L-cysteine (Ado-Cys) and 4-

5 Bromomethyl-7-methoxycoumarin (Br-Mmc) were dissolved in ethyl acetate in a molar ration of 1:3. The final volume was 25 ml (ca, 1 mg Ado-Cys). After addition of 200 mg of finely powdered K_2CO_3, the solution was refluxed for 1 hour using a ml-refluxer at 80°C. After cooling, the reaction solution was injected into a C18 column (Econosphere, C18, 7x300 mm, Alltech, Dearfield, IL) for separation using HPLC. The fluorescently labeled Ado-Cys was eluted by a

10 linear gradient of methanol; water from 20 to 100% in 30 min. The flow rate was 2 ml/min.

Method 4

Ado-Cys was dissolved in carbonate buffer, pH 9.0 in 1 mM concentration. Fluorescein isotiocyanate (FITC) (PcPierce, Rockford, IL) was dissolved in DMSO in 5 mM concentration, and diluted to 1 mM with carbonate buffer, pH 9.0. Equal volumes of Ado-Cys and FITC in

15 carbonate buffer were mixed and incubated in room temperature for 1 hour. The Ado-Cys-FITC conjugate was then isolated by HPLC using a C18 column (Econsphere, C18, Alltech, Deerfield, IL). The elution was monitored at UV 260 nm and fluorescence at Ex484 nm and Em520 nm. The mobile phases were water and methanol in a linear gradient from 0 to 80% of methanol in 35 min.

Coating mutant SAH hydrolase on microtiter well (96 well plate)

20 Mutant SAH hydrolase (F302S) was coated on flat-bottomed 96 well plate (Dynex Technologies, Chantilly, Virginia). 200 μl of 20 μg/ml of F302S mutant hydrolase in 50 mM sodium phosphate buffer, pH 7.6. was added to each well. After incubation at 4°C overnight, the plate was emptied by inversion. After blocking with 0.5% BSA, the plate was then washed three times with 10 mM PBS containing 0.1 NaCl and 0.05% of Tween 20. After inversion and tapping,

25 the plate was stored at 4°C before use.

Preparation of standard samples and chemical reagents

1. Construction of a standard Hcy curve

Human albumin (Fraction V powder, Sigma) was dissolved in PBS in a protein concentration equal to that of human plasma. To 10 ml of the albumin was added 4 ml of 1% tri-n-
5 butylphosphine (TBP). The mixture was incubated at room temperature for 15 min. followed by gel filtration through a size exclusion column (Sephacryl-S100, 2 x 90 cm). The albumin protein concentration was normalized to human plasma concentration using protein concentrator (Bio-Rad). The protein concentration was determined by Bradford reagent (Bio-Rad). A series of known concentration of L-homocysteine and L-homocystine were spiked into the TBP-treated
10 human albumin in a final concentrations ranging from 0 to 50 μM. After incubation at 37°C for 1 hour, the L-homocysteine spiked albumin and L-homocystine albumin were aliquoted in 70 μl/tube as standard samples, and stored at -20°C before use.

2. Wild type SAH hydrolase solution

The wild type SAH hydrolase (20 mU/50 μl) was dissolved in 50 mM phosphate buffer, Ph
15 7.2, containing 1 mM EDTA, 0.25 mM Ado and 1 mg/ml of BSA.

3. Tri-n-butylphosphine (TBP) solution

Tri-n-butylphoshine (Sigma) was dissolved in dimethylformamide (DMF) to 1% concentration.

4. Fluorophore-labeled Ado-Cys solution

20 Br-Mmc-labeled Ado-Cys or FITC-labeled Ado-Cys was dissolved in 50 mM phosphate buffer, pH 7.2, in a concentration of 0.5 mM.

5. SAH hydrolase inhibitor solution

Neplanocin A (natural product), an inhibitor of SAH hydrolase, and a substrate of adenosine deaminase, was dissolved in 50 mM phosphate buffer, pH 7.2. The inhibitor solution
25 (50 μM) was used in an enzyme to inhibitor ratio of 1:1.5.

6.   Multi-enzyme solution

Adenosine (0.2 U/μl), nucleoside phosphorylase (0.2 U/l) and xanthine oxidase (0.2 U/μl) were dissolved in 50 mM potassium phosphate buffer, pH 7.2. All the enzymes were from Sigma.

7.   Washing solution

5   The plate washing solution contains of 10 mM PBS, pH 7.2, 0.1 M NaCl, and 0.05% Tween 20.

EXAMPLE 4

Assays of Hcy using the mutant SAH enzyme

Plasma Hcy assay procedure 1

Step 1.   Conversion of Hcy to SAH

To 50 μl of plasma sample in microcentrifuge tube or in uncoated 96-well plate was added 20 μl of 1% TBP and 50 μl of the wild type SAH hydrolase solution. After incubation at 25°C for 15 min, 20 μl of the enzyme inhibitor solution was added to the reaction mixture, and incubated for 10 min. to inactivate SAH hydrolase.

Step 2.   Removal of remaining Ado and enzyme inhibitor

To the solution in Step 1 was added 30 μl of the multi-enzyme solution, and incubated for 15 min at room temperature.

Step 3.   Trapping the formed SAH onto the mutant SAH hydrolase

150 μl solution in Step 2 was transferred to a microtiter well pre-coated with mutant SAH hydrolase. After 30 min. incubation at room temperature, the solution was emptied by inversion.

Step 4.   Washing

The plate from Step 3 was washed three times with the washing solution followed by inversion and tapping.

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Step 5. Binding of fluorophore-labeled Ado-Cys to the mutant enzyme

100 µl of the fluorophore-labeled Ado-Cys or fluorophore-labeled Ado-5' ester was added to the microtiter well in Step 4. After 20 min. incubation at room temperature, the plate was washed three times with the washing solution.

5 **Step 6. Detection of the mutant SAH hydrolase-bound fluorophore-labeled Ado-Cys**

To the microtiter well from Step 5, 200 µl of 50 mM phosphate buffer, pH 7.2, was added, and the plate was read for fluorescence using a plate reader (Molecular Devices, fmax). The plasma Hcy concentration was calculated from the standard curve constructed under the same

10 conditions.

Alternative Hcy assay

Alternatively, the Hcy assay can also be performed by pre-coating SAH on microtiter well, and using fluorophore-labeled mutant SAH hydrolase for competition binding assay. The details are described as follows:

15 **1. pre-coating SAH on microtiter well**

SAH was conjugated to polylysine by activating the carboxylic group of SAH with PCl_3 at 50°C. The SAH-polylysine conjugate was purified by HPLC, and dissolved in 0.1 M carbonate buffer, pH 9.6. 300 µl of 100 µg/ml SAH-polylysine solution was added to each well, and incubated at 37°C for 6 hours. The plate was then washed three times with washing solution

20 containing 10 mM PBS, 0.1 M NaCl and 0.05% Tween 20. After inversion and tapping, the plate was stored at 4°C before use.

2. Fluorophore-labeled mutant SAH hydrolase

Mutant SAH hydrolase (*e.g.*, F302S) was specifically fluorescence labels on Cys421, an non-essential cysteine residue which is located on the surface of the protein that is not involved in

25 substrate binding and catalysis. Cys421 residue is readily accessible by thiol reactive molecules, and can be modified without effecting the binding affinity of the enzyme. Thiol specific reactive probes such as 7-diethylamino-3(4'-maleimidylphenyl)-4-methylcoumarin (CPM) can specifically

label protein thiols. Mutant SAH hydrolase (F302S) (0.5 mg/ml) in 50 mM phosphate buffer, pH 7.2, was incubated with 2 mM of adenine to protect other thiols in the substrate binding site, followed by addition of CPM to final concentration of 50 μM. The reaction mixture was incubated at room temperature for 30 min. followed by gel filtration on a size exclusion column (Sephacryl S-300, 4.5mmx60cm) to remove adenine and excess CPM. The CPM-labeled F302S mutant SAH hydrolase (2 mg/ml) was kept in 50 mM phosphate buffer containing 20% glycerol at -20°C. The comparison of Km (SAH) and Kcat (SAH) for wild type and mutant F302S is shown below in Table 11.

Table 4. **Comparison of kinetic constants between mutant and wild type SAH hydrolases**

Enzyme	Km (SAH)	Kcat (SAH)
wild type	7.9 μM	3.8 S^{-1}
F302S	1.0 μM	0.1 S^{-1}

Plasma Hcy assay procedure 2

Step 1. Conversion of Hcy to SAH

To 50 μl of plasma sample in microcentrifuge tube or in uncoated 96-well plate was added 20 μl of 1% TBP and 50 μl of the enzyme inhibitor solution was added to the reaction mixture, and incubated for 10 min. to inactivate SAH hydrolase.

Step 2. Removal of remaining Ado and enzyme inhibitor

To the solution in Step 1 was added 30 μl of the multi-enzyme solution, and incubated for 15 min. at room temperature.

Step 3. Competition binding of SAH to the Mutant SAH hydrolase

One hundred μl of the solution from Step 2 was transferred to a microtiter well pre-coated with polylysine-SAH conjugate to which 150 μl of the fluorophore-labeled mutant SAH hydrolase was added. After incubation at room temperature for 30 min., the plate was inverted and tapped followed by three times of washing with the washing solution.

Step 4. Detection of the fluorophore-labeled mutant SAH hydrolase bound to the microtiter well

To the plate from Step 3 was added 200 μl of 10 nM PBS, and the plate was read by a plate reader (Molecular Devices, fmax) at Ex390 nm and Em460 nm. The plasma concentration of Hcy was calculated from the standard curve constructed under the same conditions with the standard samples.

The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.